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Cytological Dissection of the Triticeae Chromosomes by the Gametocidal System

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Abstract

Large-sized and complex genomes of Triticeae species hamper assembling their sequenced data. When introduced into common wheat, some alien chromosomes called the gametocidal (Gc) chromosomes induce chromosomal breakage resulting in the generation of deletions and translocations. Thus induced deletions were established as the deletion stocks in common wheat. This Gc system is also effective in inducing chromosomal breaks in Triticeae chromosomes added to common wheat. Induced aberrant chromosomes, such as deletions and translocations between wheat and alien chromosomes, can be identified by chromosome banding and fluorescence in situ hybridization, and they can be established in common wheat, called dissection lines. Thus, the dissection of single chromosomes of Triticeae species can be achieved by the gametocidal system.

Key Words: Triticeae; common wheat; rye; barley; C-banding; N-banding; FISH; GISH; Gametocidal chromosome; alien addition; deletion; translocation; dissection line.

1. Introduction

Triticeae, a tribe in the grass family Poaceae, contains major crop genera including wheat , rye and barley. Those genera contain diploid, allotetraploid and/or allohexaploid genomes with the basic chromosome number x=7. Most of the Triticeae species have huge genome sizes, e.g. bread wheat or common wheat (*Triticum aestivum*, 2n=6x=42, 1C=16979 Mbp), rye (*Secale cereale*, 2n=2x=14, 1C=8110 Mbp) and barley (*Hordeum vulgare*, 2n=2x=14,

1C=5439 Mbp), as compared with those of rice (1C=490 Mbp) and Arabiidopsis (1C=157 Mbp) *(1)*. This large genome size has been an obstacle to complete sequencing of the Triticeae genomes.

The polyploid plants species have tolerance of aneuploidy to some extent. Especially, the hexaploid nature allowed a wide variety of aneuploid lines to be established in common wheat, i.e. monosomics, nullisomics, nullisomic-tetrasomics and telosomics *(2, 3, 4)*. In a sense, these aneuploids, which lack specific chromosomes or chromosome arms, dissected the wheat genomes, and have been used to allocate genes and DNA markers to the specific missing chromosomes or chromosome arms *(5)*.

Many Triticeae species were successfully crossed with common wheat and the F_1 hybrids were backcrossed to common wheat to produce wheat-alien chromosome addition and substitution lines *(5)*. In these lines the genomes of such Triticeae species are dissected into single chromosomes or chromosome arms in the genomic background of common wheat. The presence of alien chromosome-specific genes and DNA markers would be indicated from the phenotypes and marker profiles of the alien addition or substitution lines, respectively.

Sub-arm dissection of the chromosome would undoubtedly be more useful in genome analysis. There are two genetic systems that induce chromosomal rearrangements lacking part of chromosome arms, namely, the homoeologous pairing (*Ph*) system and the gametocidal (Gc) system. The *Ph* system has been used to generate recombination between wheat and alien chromosomes by inducing homoeolgous chromosome pairing *(6)*. The Gc system involves Gc chromosomes introduced into common wheat from various species of the genus *Aegilops*, and the Gc chromosome induces random chromosomal

breakage resulting in the generation of deletions and translocations *(7, 8)*. Fig. 1 shows how the Gc chromosome induces chromosomal structural changes: When introduced into common wheat, it induce chromosome breakage only in gametes without the Gc chromosome. The Gc system was successfully used in the production of deletion stocks of common wheat *(9)*, and the deletion stock were used in the cytological chromosome mapping of DNA markers, such as RFLPs *(10)* and ESTs *(11)*. The Gc system has also been proved to be effective in inducing chromosomal aberrations in rye and barley chromosomes added to common wheat *(12, 13)*. It is most probable that the Gc system would work for other alien chromosomes of Triticiae species, such as *Agropyron elongatum*. In the following is described the procedure how to use the Gc gametocidal system to dissect alien chromosomes added to common wheat, with examples of rye and barley chromosomes as targets of dissection. Some cytological protocols are also described because efficient and reliable chromosome identification is crucial to the successful application of the Gc system.

2. Materials

2.1. Chromosome Preparation

1. Fixative: 1 part glacial acetic acid, 3 parts ethanol (95 to 99%). Store at room temperature, not necessary to be fresh.
2. Aceto-carmine stain solution : Dissolve 1 g carmine powder (Merck) in 100 ml 45% acetic acid and boil for 24 h, using a reflux condenser to prevent the solution from being boiled dry. Transfer to a bottle without filtration and store at room temperature and store at room temperature. Use the top clear layer.

2.2. Chromosome Banding

1. Staining solution: Giemsa's stain solution or Wright's stain solution are available from different companies. Wright's Stain Solution (Muto Pure Chemicals Co. LTD., Japan) is one of the recommended stain solutions (*see* **Note 1**). Store at room temperature.
2. Phosphate buffer: Prepare a stock solution with 0.1 *M* Na_2HPO_4 (14.2 g/1000 mL) and 0.1 *M* KH_2PO_4 (13.6 g/1000 mL).
3. 5% Ba $(OH)_2$ solution: Place 50 g $Ba(OH)_2$ in a glass container and pour hot tap water up to 1000 mL. The container should be stopped tightly for storage at room temperature.
4. 2X SSC: Prepare a 20X SSC stock solution with 3 *M* NaCl and 0.3 *M* $C_6H_5Na_3O_7 \cdot 2H_2O$. Dilute tenfold with distilled water for use.

2.3. Fluorescence In Situ Hybridization (FISH)

1. 0.15 N NaOH/ethanol solution: Dissolve 6 g NaOH in 1000 mL 70% ethanol. Store at room temperature.
2. FISH probe: Using DIG-High Prime (Roche Diagnostics) or Biotin-High Prime (Roche Diagnostics), label total genomic DNAs from alien species and PCR-amplified subtelomeric repeat sequences of alien chromosomes, such as pSc200 *(14)* and HvT 01 *(15)*. Store the labeled probes at 4°C. FISH using total genomic DNA probes is called GISH.
3. ISH solution: Prepare a solution with 50% (v/v) formamide and 5% (w/v) dextran sulfate in 2 × SSC. Store at 4°C.
4. Hybridization mixture: Mix the labeled probe (1 part) and ISH solution (19 parts) (*see* **Note 2**). Heat the mixture at 90–100°C for 8–10 min and immediately freeze it until use.
5. Detection mixture: Anti-Digoxigenin-Fluorescein, Fab fragments (Roche Diagnostics) for Dig-labeled probe and Streptavidin-CY3 (invitrogen) for Biotin labeled probe diluted as

recommended by the manufacturer (*see* **Note 3**). Store at 4°C or at –20℃ for longer storage (do not repeat "thaw" and "freeze").

6. Counter staining solution: DAPI (4', 6-diamidino-2-phenylindole) (Roche Diagnostics) diluted in an anti-fade solution as recommended by the manufacturer.

2.4. Wheat stocks

1. Gametocidal lines: Addition lines of common wheat cv. 'Chinese Spring' carrying a gametocidal chromosome, 2C or $3C^{SAT}$ *(8)* (*See* **Note 4**).
2. Nullisomic-tetrasomic lines: Aneuploids of 'Chinese Spring' that lack one chromosome pair and have four doses of a homoeologous chromosome compensating for the missing chromosome pair.
3. Alien addition lines: Addition lines of common wheat cv. 'Chinese Spring' carrying single alien chromosomes derived from rye, barley and other Triticeae species.

The gametocidal and nullisomic-tetrasomic lines and many alien chromosome addition lines can be obtained from the National BioResource Project (NBRP) (http://www.shigen.nig.ac.jp/wheat/komugi/top/top.jsp).

3. Methods

3. 1 Chromosome preparation

1. Place root tips in small vials filled with distilled water and immerse them in ice water for 16–20 h to collect metaphase cells.

2. Fix the root tips in the fixative for 1 day, stain them in the aceto-carmine solution for 1–2 h, place them back in the original fixative, and store them for 2–3 days for chromosome banding and for 3–6 days for FISH. All procedures are at room temperature. Thus fixed and stained root tips can be stored at –20℃ until use.
3. Stain the fixed and stained root tips again in the aceto-carmine solution for 10–20 min.
4. Make chromosome preparations by the squash method from the stained root tips and immediately store them at –70℃ or below until use.
5. Remove a cover slip quickly from the frozen slide, using a razor blade, immerse the slide in 45% acetic acid at 40–45℃ for 2–3 min, and air-dry the slide at room temperature. The air-dried slide can be used immediately or stored in an airtight container at –20℃ up to several months

3. 2 Chromosome banding

N-banding

1. Incubate the air-dried slide (see ***3.1***) in 1 *M* NaH_2PO_4 solution for 1.5 min at 92–95℃.
2. Wash the slide briefly with hard tap water (*see* **Note 5**).
3. Place the wet slide into the staining solution at room temperature until appropriate staining is achieved (usually about 2 h).

C-banding

1. Place the air-dried slide (see ***3.1***) in a container with a lid.
2. Pour 5% $Ba(OH)_2$ solution into the container, put the lid on it, and keep it for about 5 min at room temperature.

3. Take out the slide, quickly wash it with hard tap water, and incubate in 2x SSC for 10 min at 42–45℃.
4. Wash the slide briefly with hard tap water.
5. Place the wet slide into the staining solution at room temperature until appropriate staining is achieved (usually about 50 min).

Microscopic observation

1. Wash the stained slide briefly with hard tap water.
2. Air-dry the slide using a puffer (a camera tool).
3. Mount the slide in immersion oil with or without a cover slip (*see* **Note 6**).

Fig. 2 shows a C-banded mitotic metaphase cell of rye (a) and N-banded mitotic metaphase cell of barley (b).

3.3 FISH/GISH

1. Immerse the air-dried slide (see ***3.1***) in the 0.15 N NaOH/ethanol solution for 5 min at room temperature.
2. Transfer the wet slide into a series of two 70% and one 99% ethanol each for 3 min at room temperature.
3. Dry the slide quickly with a puffer.
4. Apply the denatured hybridization mixture (10 µL per slide) and place a cover slip on it and incubate the slide in a moistened chamber for 6 h–24 h at 30℃ (*see* **Note 7**).

5. Remove the cover slip with a pair of forceps (when the cover slip is firmly stuck on the slide, do not remove it by force, but let the cover slip fall down by itself by dipping it in 2 × SSC) and immerse the slide for 3 min in 2 × SSC at room temperature.
6. Wash the slide briefly with distilled water and blow off water using a puffer.
7. Apply the detection mixture (10 μL per slide) and place a cover slip on it and incubate the slide in a wet chamber for about 1 h at 30℃.
8. Remove the cover slip with a pair of forceps (when the cover slip is firmly stuck on the slide, do not remove it by force, but let the cover slip fall down by itself by dipping it in 2 × SSC) and wash the slide briefly with distilled water and blow off water using a puffer.
9. Apply the counter staining solution (5 μL per slide) and put a cover slip on it for fluorescence microscopic observation.

FISH/GISH after Chromosome Banding

1. After recording the images of chromosome banding, wash off immersion oil from the slide by dropping a mixture of xylene/99% ethanol (1:1) onto the slide three times and blowing off the mixture using a puffer.
2. Dip the slide in 70％ ethanol for 10 min or longer and in 99％ ethanol for 5 min or longer at room temperature.
3. Air-dry the slide using a puffer.
4. Treat the slide as described in ***3.3***.

3.4 Dissection of Treticeae Chromosomes

Deletion Lines of Common wheat

The deletion stocks of common wheat cultivar ‘Chinese Spring’ has been produced from deletions induced mostly by the 2C chromosome and screened by C-banding *(18).* Fig. 3 shows a C-banded mitotic metaphase cell of a Chinese Spring plant with three deletions. At present about 350 deletion-homozygous lines are available from NBRP, but the number of deletion stocks for each chromosome is at most only 36 for1B. Although an unlimited number of deletions can practically be produced by the same way, but the C-banding, which needs skill and is time consuming, limit us to obtain hundreds of deletions for each chromosome. However, the use of nullisomic-tetrasomic lines and PCR-based chromosome-specific markers would enable us to screen chromosome-specific deletions in a large scale. The procedure is as follows.

1. Cross the disomic 2C line with euploid ‘Chinese Spring’ to obtain monosomic 2C plants.
2. Cross the monosomic 2C plants as the female parent with one of the nullisomic-tetrasomic lines.
3. Grow the progeny plants from more or less shriveled seeds, which are liable to have chromosomal aberrations. Plump seeds tend to have the Gc chromosome and no chromosomal aberration.
4. Conduct PCR analysis of the plants using PCR markers specific to the chromosome missing in the nullisomic-tetrasomic line that was used to pollinate the monosomic 2C plants. The most distal markers on both chromosome arms are the most suitable for the detection of terminal deletions of the chromosome.
5. Select plants missing any of the chromosome-specific markers. Those plants can be regarded as lacking part of the critical chromosome in hemizygous condition.

Developing Alien Addition Lines carrying a Gc chromosome

1. Cross the Gc lines with an alien addition line of common wheat.
2. Backcross the F_1s to the alien addition line.
3. Select plants (2n=45) disomic for the alien chromosome and monosomic for the Gc chromosome by C- or N-banding and GISH. An example with the 45-chromosome constitution is shown in Fig. 4.
4. Backcross the 45-chromosome plants to euploid 'Chinese Spring' and grow the progeny plants from more or less shriveled seeds. Plump seeds tend to have the Gc chromosome and no chromosomal aberration.
5. Harvest root tips and DNA from the progeny plants for screening.

Cytological Screening for and Characterization of Aberrant Alien Chromosomes

1. Check the chromosome constitutions of the progeny plants by FISH/ GISH. The FISH probes of alien species-specific subtelomeric sequences, such as HvT 01 for barley chromosomes and pSc200 for rye chromosomes, are useful in detecting aberrations of the alien chromosomes. Fig. 5 shows examples of aberrant alien chromosomes identified by FISH/GISH.
2. When two alien chromosomal segments are present in a plant as shown in Fig. 5a, backcross the plant, and select plants with the single alien chromosomal segments plants among the progeny.
3. Thus established lines with single alien chromosomal segments are called dissection lines of the alien chromosome.
4. For some aberrant alien chromosomes, the chromosome arm where the breakpoint is located can be known from the FISH/GISH image (Fig. 5b). For some aberrant alien

chromosomes, however, sequential C-(or N-) banding and FISH/GISH is necessary to Identify the chromosome arm where the breakpoint is located (Fig. 5c).

PCR Screening for Deletions of Alien Chromosomes

With the use of the most proximal marker among those mapped to an alien chromosome, terminal deletions of the alien chromosome can be detected by PCR in the progeny of the 45-chromosome plants backcrossed to euploid 'Chinese Spring'. Although some deletions can not be detected by PCR when the deleted segments are translocated to wheat chromosomes, the PCR screening is the most efficient way of large-scale screening for deletions of alien chromosomes added to common wheat.

Deletion mapping of DNA Markers using Dissection Lines of Alien Chromosomes

1. Collect alien-chromosome-specific markers such as RFLPs, SSRs and ESTs.
2. Check the polymorphism of the markers between the alien chromosome addition line and euploid 'Chinese Spring'.
3. Examine the presence or absence of the polymorphic markers in an array of dissection lines.
4. Arrange the results into a matrix and construct a cytological map of the alien chromosome with the DNA markers.
5. The DNA markers can be used, instead of FISH/GISH, in screening for plants carrying aberrant alien chromosomes in the progeny of dissection lines *(12, 13)*. The most proximal DNA markers on both chromosome arms make universal makers to check the presence of critical aberrant alien chromosomes in dissection lines.

4. Notes

1. Choice of stain solutions is crucial to successful chromosome banding. Therefore, different stain solutions from different manufacturers should be tried.
2. The proportion of the labeled probe to ISH solution should be minimized to attain the least FISH/GISH background signals but sufficient FISH/GISH signals for observation and photography.
3. The detection mixture can be diluted much more than the manufacturer recommends.
4. Both Gc chromosomes induce deletions and translocation. It seems, however, that the Gc action of $3C^{SAT}$ is weaker than that of 2C and that $3C^{SAT}$ induces more translocations than 2C does.
5. In Kyoto, Japan, 0.65 g $CaSO_4 \cdot 2H_2O$ is added to 1000 mL tap water to make it hard water. The use of distilled water or soft tap water would deteriorate differential staining of chromosomes.
6. A special 100x objective that does not require a cover slip is available (OLYMUPUS, MPLAPON 100XO), but ordinary 100x objectives can be used without a cover slip.
7. Thanks to the lower temperature 30℃ for hybridization, GISH without unlabeled blocking DNA generates as satisfactory signals as those generated by other GISH protocols using 37℃ for hybridization.

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Figure legends

Fig. 1. Schematic illustration of the action of the Gc chromosome. See the text for details.

Fig. 2. A C-banded mitotic metaphase cell of 'Prolific' rye (a) and a N-banded mitotic metaphase cell of 'Shinebisu' barley (b). The chromosome designations of rye and barley are based on the karyotypes in previous studies *(16, 17)*. The identification of chromosomes 2R, 3R and 7R is not clear. N-banding and C-banding patterns are basically the same in barley,

but N-banding differentiates no terminal heterochromatic bands in rye as C-banding does.

Fig. 3. A C-banded mitotic metaphase cell of a common wheat plant carrying deletions. Note this plant has three different deletions of 5A, 7A and 5B (pointed with arrows), and is partially trisomic for 5B.

Fig. 4. A C-banded mitotic metaphase cell of a 45-chromosome plant of common wheat disomic for 2H and monosomic for 2C.

Fig. 5. The FISH/GISH images of reciprocal translocations involving 1R chromosome of ‘Imperial’ rye (a) and of a translocation involving 5H chromosome of ‘Betzes’ barley (b), and the sequential C-banding and FISH/GISH image of a deletion of 7H chromosome of ‘Betzes’ barley (c). The brightest parts (green in color) represent FISH signals of pSc200 repeats in (a) and HvT 01 repeats in (b) and (c), the less bright regions (pink in color) show GISH signals of rye chromatin in (a) and of barley chromatin in (b) and (c), and the least bright regions (blue in color) shows wheat chromosomes.

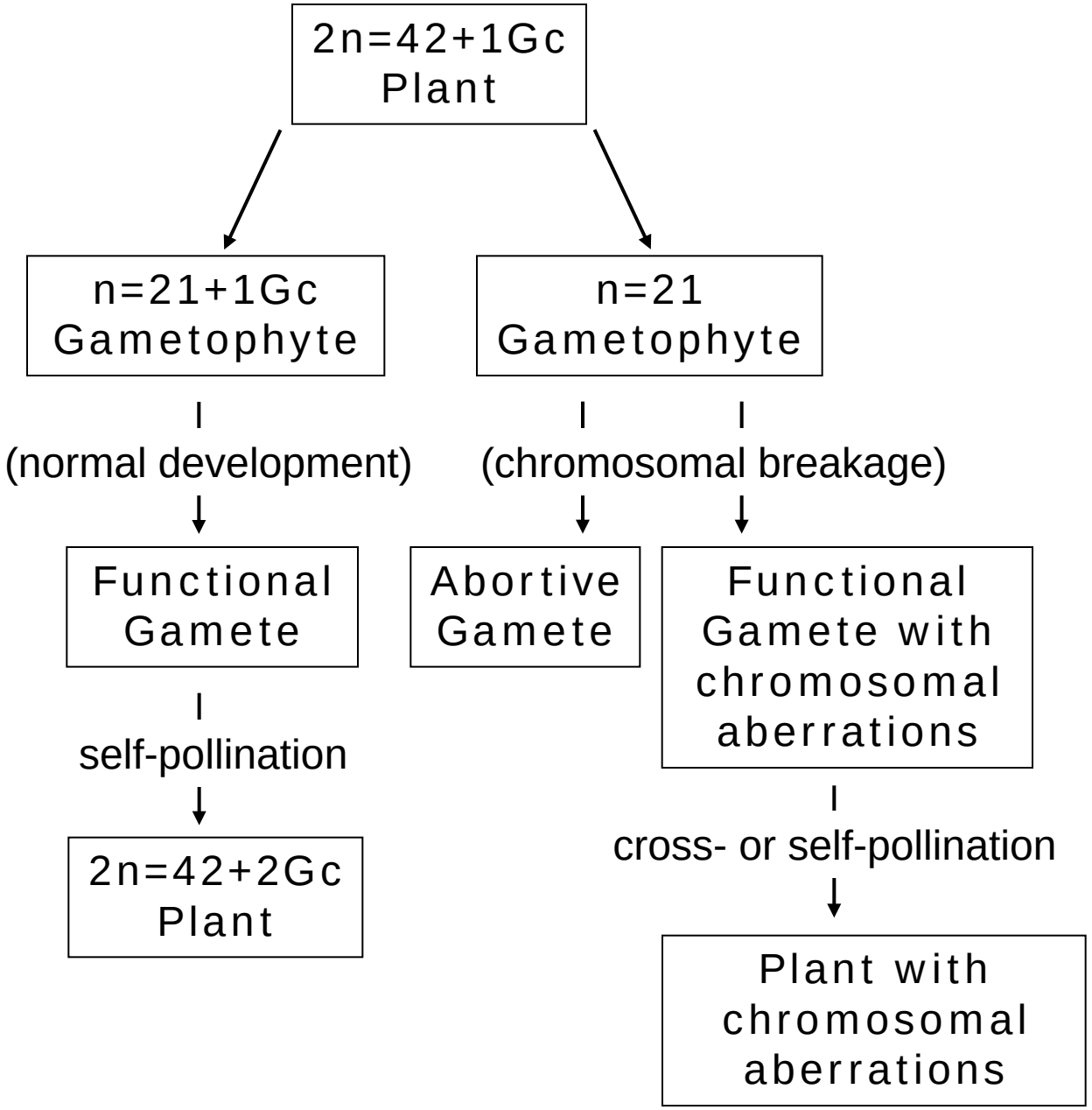
2n=42+1Gc Plant
n=21+1Gc Gametophyte
n=21 Gametophyte
(normal development)
(chromosomal breakage)
Functional Gamete
Abortive Gamete
Functional Gamete with chromosomal aberrations
self-pollination
cross- or self-pollination
2n=42+2Gc Plant
Plant with chromosomal aberrations

Fig. 1

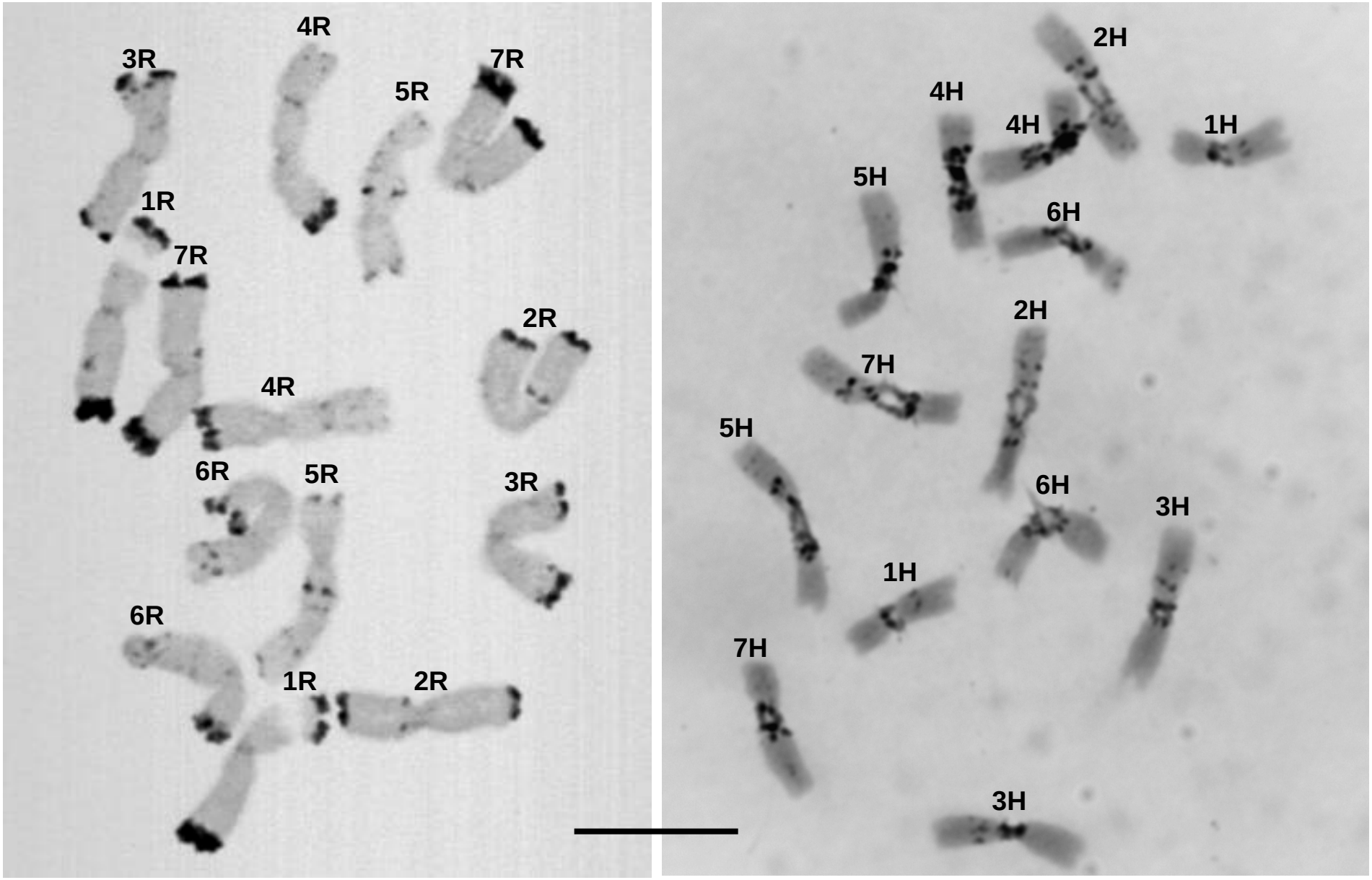
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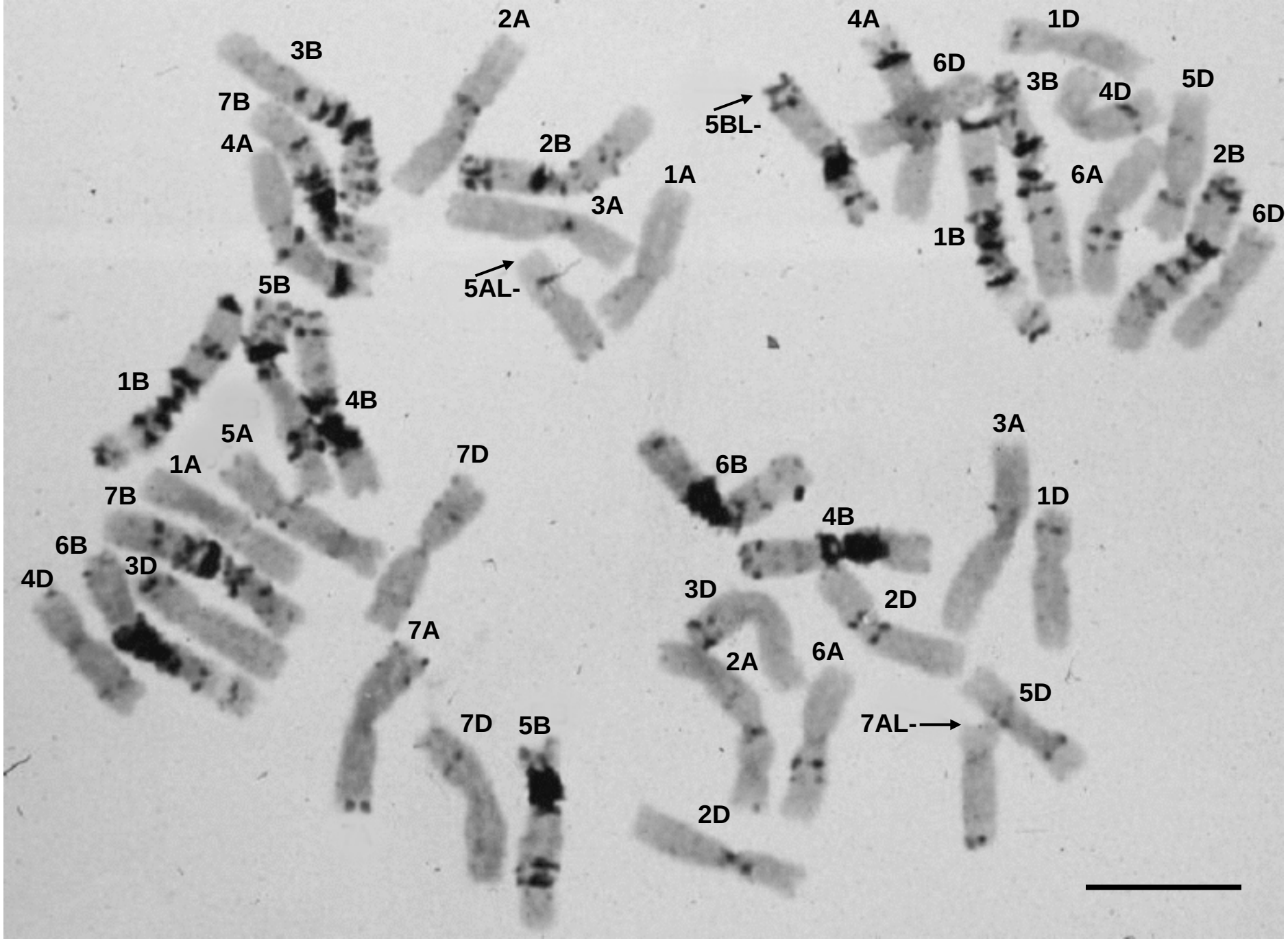
Fig. 2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5B
5BL-
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Fig. 3

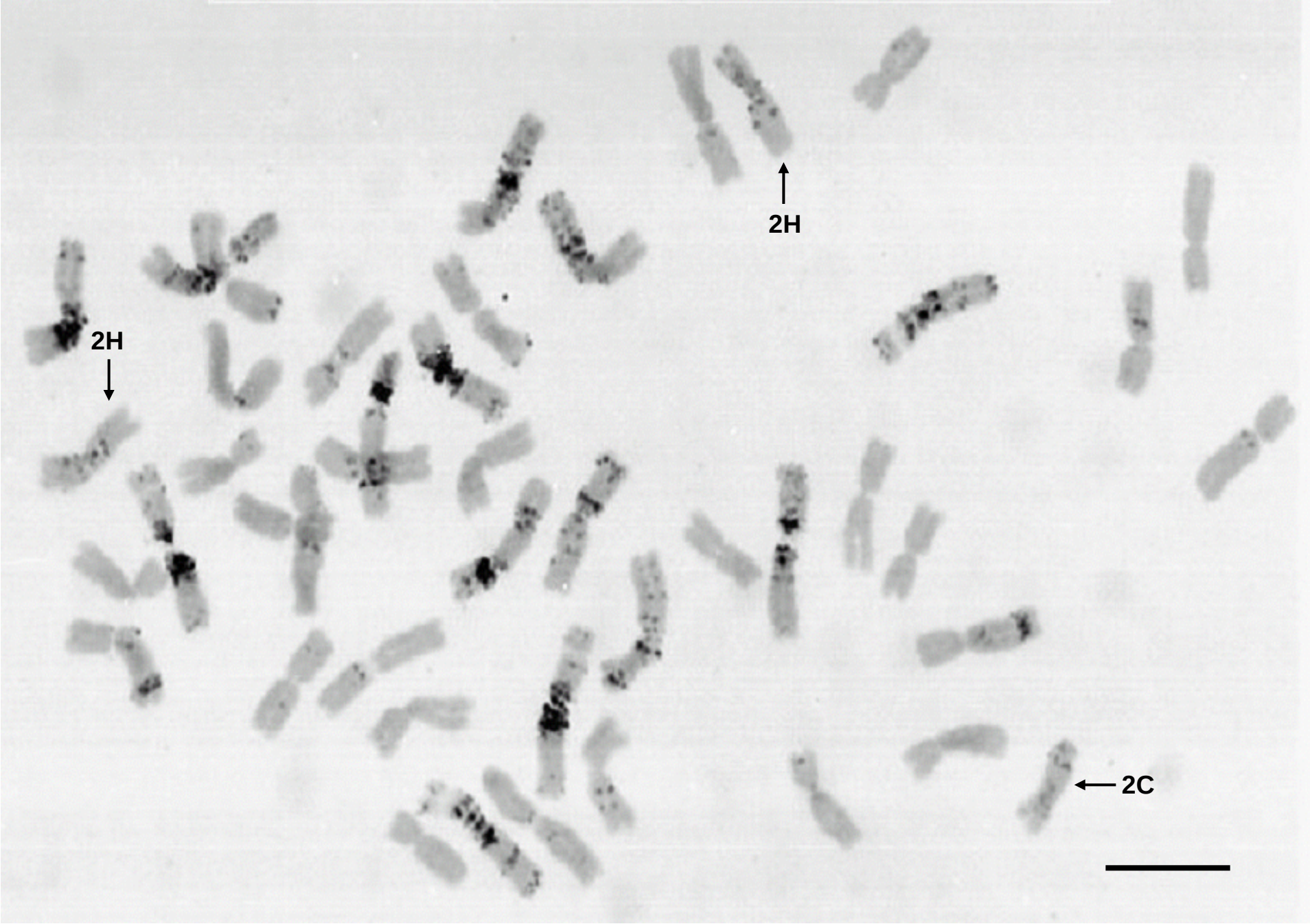

Fig.4

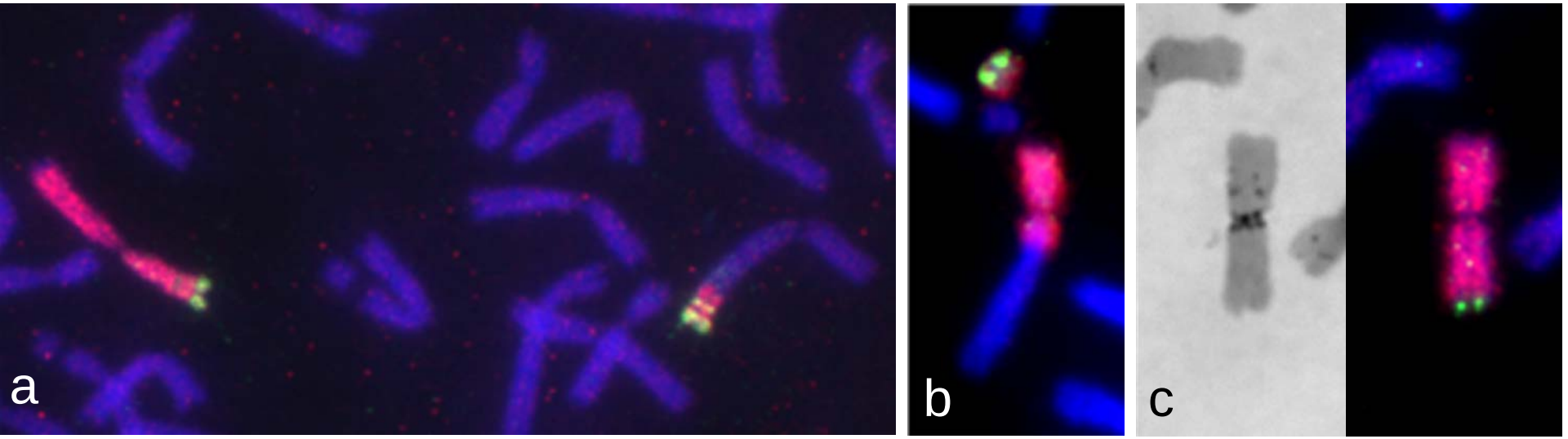

Fig. 5